The Fractionation of Nitrogen and Oxygen Isotopes in Macroalgae during the Assimilation of Nitrate

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Abstract

In order to determine and understand the stable isotope fractionation of ^{18}O and ^{15}N manifested during assimilation of NO_3^- in marine macro-benthic algae, two species (*Ulva* sp. and *Agardhiella* sp.) have been grown in a wide range of NO_3^- concentrations (2-500 μM). Two types of experiments were performed. The first was one in which the concentration of the NO_3^- was allowed to drift downward as it was assimilated by the algae, between 24 hour replacements of media. These experiments proceeded for periods of between seven and ten days. A second set of experiments maintained the NO_3^- concentration at a low steady state value by means of a syringe pump. The effective fractionation during the assimilation of the NO_3^- was determined by measuring the $\delta^{15}N$ of both the (i) new algal growth, and (ii) residual NO_3^- in the free drift experiments after 0, 12, 24 and 48 hours. Modelling these data show that the fractionation during assimilation is dependent upon the concentration of NO_3^- and is effectively zero at concentrations of less than ~ 2 μM. The change in the fractionation with respect to concentration is the greatest at lower concentrations (2-10 μM). The fractionation stablizes between 4-6‰ at concentrations of between 50 and 500 μM. Although the $\delta^{18}O$ and $\delta^{15}N$ values of NO_3^- in the residual solution were correlated, the slope of relationship also varied with respect to NO_3^- concentration, with slopes of greater than unity at low concentration. These results suggest shifts in the dominant fractionation mechanism of ^{15}N and ^{18}O between concentrations of 1 and 10 μM NO_3^-. At higher NO_3^- concentrations (> 10-50 μM), fractionation during assimilation will lead to $\delta^{15}N$ values in algal biomass lower than the ambient NO_3^- and ^{15}N enrichments in the residual NO_3^-.

1 Introduction

Nitrogen availability is an important factor in controlling algal growth in marine environments, representing a limiting nutrient throughout much of the global ocean (Dugdale and Wilkerson, 1986). In many studies, information on nitrogen sources and its cycling has been obtained by examining the ratio of the stable isotopes of nitrogen (^{14}N and ^{15}N) as well as oxygen (^{18}O and ^{16}O) in the case of NO_3^-. Isotope ratios are expressed using the conventional 'delta' notation ($\delta^{15}N$ or $\delta^{18}O$) in parts per thousand (‰) deviation from the atmospheric N_2 standard or, in the case of oxygen, from Vienna standard mean ocean water (VSMOW). During cycling of NO_3^-, isotope fractionation takes place, as quantified by the associated fractionation factor (α). For algal NO_3^- uptake, α can be calculated using equation 1. The term epsilon (ε) is also commonly used and is related to α by equation 2.

$$\alpha = \frac{\frac{15}{14}algae}{\frac{15}{14}Solution} \quad (1)$$

$$\varepsilon = (\alpha - 1) * 1000 \quad (2)$$

The term ε can refer to fractionation of either ^{15}N ($^{15}\varepsilon$) or ^{18}O ($^{18}\varepsilon$) relative to the more abundant isotope of the element. In some of these processes, such as the fixation of atmospheric nitrogen, no significant isotopic fractionation takes place ($^{15}\varepsilon \sim 0.0$‰) (Hoering and Ford, 1960) and consequently the $\delta^{15}N$ of N_2 fixing organisms is similar to that of atmospheric N_2 (0‰ by convention). In other processes, such as the denitrification of NO_3^-, $^{15}\varepsilon$ values reach values higher than 20‰ (Barford et al., 1999; Delwiche and Steyn, 1970;

Granger et al., 2006; Miyake and Wada, 1971), leading to large increases in the $\delta^{15}N$ of the residual reservoir of NO_3^-. While the $\delta^{15}N$ of microalgae has been studied in order to understand its use as a paleoceanographic proxy (Altabet, 1989; Altabet et al., 1991; Haug et al., 1998; Sigman et al., 2003), variations in the $\delta^{15}N$ of macroalgae have also been widely used as possible indicators of anthropogenic influences (Carballeira et al., 2013; Costanzo et al., 2001; Heaton, 1986). Generally speaking, nitrogen derived from sewage is isotopically enriched in ^{15}N and it has been argued that even modest enrichments of ^{15}N in macroalgae might reflect enhanced input from such sources (Lapointe et al., 2004). Other studies have shown that such enrichments could occur through normal processes including fractionation during assimilation (Lamb et al., 2012; Stokes et al., 2011) and that there are not always simple relationships between the input of anthropogenic wastes and $\delta^{15}N$ values (Viana and Bode, 2013).

Studies of isotope fractionation during the assimilation of dissolved inorganic nitrogen by marine microalgae have reported a wide range of values. In one study, reported $^{15}\varepsilon$ values ranged from 0.7 to 23‰ for the assimilation of NO_3^- by *Pheodactylum tricornutum* (Wada and Hattori, 1978), a marine diatom. Another study reported $^{15}\varepsilon$ values between 2.2 and 6.2 ‰ for 12 different marine phytoplankton cultures kept at a NO_3^- concentration of 100 μM (Needoba et al., 2003). Other research also report wide ranges in $^{15}\varepsilon$ values for both NO_3^- and NH_4^+ for a variety of different microalgae (Horrigan et al., 1990; Lajtha and Michener, 1994; Montoya et al., 1990; Wada and Hattori, 1978). At least part of these large ranges in $^{15}\varepsilon$ values probably resulted from variations in experimental conditions and are perhaps artifacts resulting from differences in aeration, light and nutrient drawdown. In addition, changing nutrient concentration might be an important controlling parameter and several studies have

shown that microalgae show varying fractionation as a function of concentration (Hoch et al., 1992; Pennock et al., 1996; Waser et al., 1998) that is likely due to changes in physiology and perhaps uptake mechanism.

In contrast to microalgae, there have been relatively few studies of ^{15}N fractionation in macroalgae. Some of these studies have relied on spiking the natural environment with high nitrate and ammonium concentrations (Teichberg et al., 2007), while others have used transplant experiments (Deutsch and Voss, 2006). Neither of these investigations reported $^{15}\varepsilon$ values for fractionation during the assimilation of NO_3^-. The study of Cohen and Fong (2005) grew the green alga *Enteromorpha intestinalis* under varying concentrations of NO_3^- and NH_4^+ and, although they did not report values for ^{15}N fractionation, they concluded that the $\delta^{15}N$ of the algae was not dependent upon concentrations of dissolved inorganic nitrogen. These experiments used a combination of increases in NO_3^- and NH_4^+ with the lower NO_3^- concentration experiments containing high amounts of NH_4^+ and vice versa. Under such experimental conditions it would have been difficult to isolate any potential concentration dependence upon fractionation manifested during assimilation. Given the possibility of a concentration dependence of ^{15}N fractionation for NO_3^- in microalgae, we revisit here whether such a dependency is found in macroalgae. We have used two different approaches over a range of different concentrations. In the first series of experiments, two species of macroalgae, *Ulva* sp., and *Agardhiella* sp, were grown over a range in nominal NO_3^- concentrations of 10, 50, 100 and 500 μM. As the algae within each culture consumed the NO_3^- in the solution, the solutions were replaced every 24 hours. These were the so-called free drift experiments. In the second set of experiments, NO_3^- levels were maintained at a low level (< 2 μM) by continual addition from a syringe pump. Hence these experiments cover

the range of NO_3^- concentrations used in most previous experiments (> 100 μM) as well as those seen under natural conditions.

2 Methods

Samples of the green algae *Ulva* sp. and the rhodophyte algae *Agardhiella* sp. were collected from cultures held at the *Aplysia* Mariculture Laboratory's algal aquaculture facility (University of Miami). These species were maintained in a system of seven, 9,000 liter fiberglass tanks supplied with filtered seawater at a rate of ~ 22 l min^{-1}. Radiant energy and temperature are monitored constantly and algal growth rates are optimized by adjusting nutrient levels weekly. These stocks are kept continually as a food source for other organisms in the facility. In preparation for these experiments the algal thalli were rinsed with filtered seawater and gently scrubbed to remove surface epiphytes. Prior to experimentation, the macroalgae were maintained within 2L flasks at 26°C and approximately 100 μmol photons m^{-2} s^{-1} for a 14-day acclimation period. During the acclimation period, filtered and autoclaved seawater was changed every 2 days, enriched to 500μM N (250 μM $NaNO_3$ and 250 μM NH_4Cl) and 44 μM KH_2PO_4, with f/2 medium supplements of B-vitamins (Vitamin B_{12}, Biotin, and Thiamine) and trace metals (Fe, Cu, Mo, Zn, Co, and Mn) (Guillard, 1975). The cultures were continually aerated throughout the incubations.

2.1 Experimental Protocol

2.1.1 Free Drift Experiments

In these experiments the effect of varied nutrient availability on the nitrogen isotopic composition of new algal growth with respect to varied NO_3^- concentration was investigated. Nominal concentrations of 10, 50, 100 and 500 μM N ($NaNO_3$) were supplied in a medium of autoclaved, filtered (0.2μm cartridge filter) seawater enriched with the same KH_2PO_4, B-vitamin, and trace metal supplements outlined for the acclimation medium (Note that the actual targeted and measured concentrations were slightly different and the values used are reported in Table 1 and 2). Subsamples of *Ulva* and *Agardhiella* (0.25-0.5g wet weight; 2.5-3.0 cm) were taken from acclimation flasks, any visible epiphytes were again removed, and the algae samples were placed in 2L flasks filled with incubation medium. The media was replaced every 24 hours at which time each algal sample was rinsed to prevent epiphyte accumulation. The experiments proceeded for a period of 7-9 days. Water samples were collected after each 24-hour period and analyzed for the concentrations of NO_3^- and NH_4^+. At the conclusion of the incubations, final accumulated biomass was weighed and as the new algal growth produced was clearly visible, material which had grown only under the experimental conditions was trimmed off (Figure 1). This material was dried (40°C 48 hours), then ground with mortar and pestle for subsequent N isotopic analyses and C:N determination. In order to examine the effect of assimilation on the $\delta^{15}N$ of residual NO_3^-, special experiments were performed in which the same water was kept in the algal cultures for periods of up to 48 hours. After 12, 24 and 48 hours water samples were taken and the $\delta^{18}O$ and $\delta^{15}N$ of the NO_3^- measured.

2.1.1 Constant NO_3^- Concentration Experiments

At low concentrations of NO_3^- (<10 μM) the algae rapidly assimilated NO_3^- and concentrations decreased to values of less than 3 μM within a few hours. In order to maintain a consistent low concentration and provide sufficient NO_3^- for the algal growth, NO_3^- was continuously added by means of a syringe pump. The rate of addition was initially determined by using the uptake rates calculated from the free drift experiments and then adjusted slightly after the analysis of the NO_3^- concentration in the experiment. In these experiments concentrations started at ~10 μM and stabilized at 3 μM throughout the growth period.

2.2 Analytical Protocol

2.2.1 Stable Isotopes

1.2.1.1 Algal biomass. The organic carbon and nitrogen content as well as the stable nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotopic composition of the algae was determined using a CN analyzer (ANCA, Europa Scientific) interfaced with a continuous-flow isotope-ratio mass spectrometer (CF-IRMS) (20-20, Europa Scientific). Prior to analysis the algae samples were dried and 3 - 6 mg were placed in tin capsules. Data obtained from the mass spectrometer provides the C/N ratio of the samples in addition to the isotopic content of the organic matter. Samples of the nutrient salts added were analyzed in a similar manner to determine the initial $\delta^{15}N$ of the medium. The $\delta^{15}N$ and $\delta^{18}O$ of the initial NO_3^- was also analyzed as dissolved inorganic nitrogen (See below). Internal laboratory standards, calibrated to VPDB and atmospheric N_2, were analyzed every ten samples and data were corrected relative to the mean

of the two nearest standards. External precision is approximately ±0.2‰ for $\delta^{15}N$ and ±0.1 for $\delta^{13}C$. The C:N ratio was calculated by comparing the integrated area of the major beams (mass 28 for N and mass 44 for C) to standards with known C:N ratios. The external precision for this method is < 0.1%.

2.1.1.2 Dissolved Inorganic Nitrogen. The $\delta^{15}N$ and $\delta^{18}O$ composition of the samples were determined using a GV IsoPrime with an external automated purge-and-trap system at the University of Massachusetts, Dartmouth, SMAST campus. The NO_3^- was converted to N_2O using Cd reduction to NO_2^- followed by azide treatment (McIlvin and Altabet, 2005). Data are reported relative to atmospheric N_2 and VSMOW for nitrogen and oxygen, respectively. Each run of NO_3^- samples consisted of one operational blank (low nutrient seawater treated with azide), three NO_2^- standards, a cadmium blank (low nutrient seawater treated with cadmium) and three NO_3^- standards (USGS 34, 35 and an internal Altabet lab standard), followed by the prepared samples. Three randomly selected samples were also prepared in triplicate to check for method and machine reproducibility. The run ended with three more NO_2^- standards, three NO_3^- standards, a Cd blank and an operational blank. Analytical precision measured from multiple determinations on standards was approximately ±0.2 ‰ for $\delta^{15}N$ and ±0.7 ‰ for $\delta^{18}O$ (NO_3^- only).

Isotopic data produced from each run were scrutinized for standard precision throughout individual runs. Samples were corrected for the small amount (~15%) of oxygen exchange that occurs between the sample and water during the conversion to nitrous oxide, fractionation due to oxygen removal, as well as the 1:1 addition of azide-N to NO_2^--N in the formation of N_2O (see McIlvin and Altabet (2005) for an in depth discussion of $\delta^{15}N$ and $\delta^{18}O$ corrections).

2.2.2 Nutrient Concentrations

Concentrations of NO_3^-, NO_2^- and NH_4^+ in the growth solutions were analyzed prior to, during and after each experiment. Nitrate and nitrite concentrations were determined by diazotization before and after reduction with cadmium (Grasshoff, 1976). Ammonium concentrations were determined with the indophenol-blue method. Note that the measured concentrations of the NO_3^- were slightly different than initial target concentrations.

3 Results

3.1 Nitrogen Isotopes in Algal Material

3.1.1 Free Drift Experiments

Results from the free drift nutrient experiments from *Ulva* and *Agardhiella* are presented in Table 1. In each of the treatments the $\delta^{15}N$ of the new algal growth during each experiment and the residual NO_3^- concentrations left in each treatment after the 24 hour incubations was determined. Although concentrations of NO_2^- and NH_4^+ were measured, none was detected. The $\delta^{15}N$ of the newly grown *Agardhiella* material decreased from 1.8 ‰ (14 μM) to 1.6 ‰ in the 50 μM treatment, to 0.7 ‰ in the 103 μM treatment and finally to -3.0 ‰ in the 485 μM experiment. Similar results were found in the experiments using *Ulva* although the $\delta^{15}N$ values were all higher (Table 1). For example, in the lowest two NO_3^- treatments, the $\delta^{15}N$ of the *Ulva* was actually more positive than that of the NO_3^- in the growth medium (Table 1).

The C:N ratios and the $\delta^{13}C$ values of the algae are included in the supplementary information.

3.1.2 Syringe Experiments

The results from all the syringe experiments are listed in Table 2. The $\delta^{15}N$ value of *Ulva* and *Agardhiella* exhibited small decreases.

3.2 Isotopic analysis of Dissolved Inorganic Nitrogen

3.2.1 Free Drift Experiments

The data from the free drift experiments are presented in Table 1 with the trend in the $\delta^{15}N$ of the NO_3^- mirroring that of the solid algae. The mean $\delta^{15}N$ and $\delta^{18}O$ values of the initial NO_3^- were +3.3 +/- 0.3‰ and +23 +/- 0.3 respectively (n=12) and as the NO_3^- was consumed, the residual NO_3^- became isotopically enriched in ^{15}N and ^{18}O. The $\delta^{15}N$ and $\delta^{18}O$ values in both *Agardhiella* sp. (r^2=0.60, n=13) and *Ulva* sp. (r^2=0.79, n=25) esperiments were positively correlated to each other exhibiting a slope close to unity for both algae species (1.1 for *Ulva* sp. and 1.17 for *Agardhiella* sp.). In the lower concentration experiments the slope increased to approximately two.

4 Discussion

In order to calculate the fractionation during assimilation, the change in the $\delta^{15}N$ and $\delta^{18}O$ of the NO_3^- and the algal tissue were modelled using a Rayleigh distillation model. In the case of N, the $^{15}N/^{14}N$ of the new algal growth (RA) at time (t) is given by equation 3, while the $^{15}N/^{14}N$ of the residual NO_3^- (R) at t is given by equation 4.

$$RAt = Ri\frac{1-f^{1/\alpha}}{1-f} \quad (3)$$

$$Rt = Rif^{(\frac{1}{\alpha}-1)} \quad (4)$$

In these equations (f) represents the fraction of the initial NO_3^- remaining, (Ri) the $^{15}N/^{14}N$ ratio of the initial NO_3^-, (Rt) and (RAt) the $^{15}N/^{14}N$ ratio of the NO_3^- and new algal growth respectively after a specific time during which (f) has been determined, and (α) the fractionation factor. The fractionation factor ($^{15}\varepsilon$) can also be calculated using the approach of Mariotti et al. (1981) which utilizes a plot of the isotopic composition of the NO_3^- with respect to ln f or ln (NO_3^-(t)/NO_3^-(i)) as in equation 5.

$$\delta t = \delta i - \varepsilon \ln f \quad (5)$$

In equation 5, ε ($^{15}\varepsilon$) is the slope of the relationship between δ^{15}Nt and ln f. The term δt = the δ^{15}N of the NO_3^- at time (t) when the concentration is equal to NO_3^- (t) and δi = δ^{15}N of the NO_3^- at the initial time when the concentration is equal to NO_3^- (i). In the free drift experiments where the δ^{15}N of the solution was sampled multiple times the δ^{15}N values were measured at various concentrations as the NO_3^- was assimilated by the algae and hence values of ln f calculated. A similar approach was used to calculate $^{18}\varepsilon$ using the δ^{18}O data.

An alternative method for calculating the fractionation factor used the measurement of the δ^{15}N of new algal tissue as a function of the expression in equation 6.

$$x = \frac{f ln f}{1-f} \quad (6)$$

As f tends to 0 (all the NO_3^- was consumed) then the $\delta^{15}N$ of the algae (δA) tended to approach the $\delta^{15}N$ of the initial NO_3^-. Hence utilizing equation 7, the slope of the relationship was equivalent to (α-1)*1000 or ε.

$$\delta At = \delta Ai - \varepsilon x \quad (7)$$

In each of the experiments the slope of the line was determined by plotting the initial $\delta^{15}N$ of the NO_3^- at a f value of zero and the measured $\delta^{15}N$ of the algae at the appropriate f value corresponding to the decrease in the concentration of NO_3^- at the end of 24 hours.

While it is possible to arrive at an estimate of fractionation using either the solid sample or the NO_3^- data, the method of measuring the $\delta^{15}N$ of the residual NO_3^- may provide a more accurate method for a number of reasons. First, in the case the measurement of the $\delta^{15}N$ of the tissue in the free drift experiments, the f factor is calculated by averaging the amount of NO_3^- utilized during a 24 hour period. This assumes that the algae grows equally throughout the 24 hour period, rather than perhaps faster when the NO_3^- concentration is high and lower as the concentration is reduced. In addtion as the concentration of NO_3^- is reduced to low concentrations the fractionation of ^{15}N will also change (see later discussion). It might be possible to model these changes, but the interpretation would be dependent upon a number of assumptions which could not be validated with the present dataset. In this regard the $\delta^{15}N$ of the tissue grown in the syringe experiments might be more reliable in providing an estimate of

fractionation as a constant amount of NO_3^- is supplied throughout the growth period and therefore Rayleigh type modelling is unnecessary. In addition, both the syringe and the free drift tissue measurements might suffer from the inability to precisely separate new and old algal tissue growth and the possibility of translocation of N bearing compounds in the algal tissue. In contrast, the $\delta^{15}N$ of the residual NO_3^- provides a direct measurement of the factionation during assimilation. While the results obtained between the two methods are similar, in cases where there are differences, we feel that the data obtained from the $\delta^{15}N$ of the NO_3^- provides the best estimate of fractionation.

4.1 Modelling

As the NO_3^- removed from the medium was balanced by algal assimilation, isotopic fractionation produced corresponding changes in both the $\delta^{15}N$ of the residual NO_3^- and the $\delta^{15}N$ of new algal growth. These data are reported in Table 1 and the fractionation factors estimated for ^{15}N (and ^{18}O when applicable) using equations 5 and 7 are reported in Table 3.

4.1.1.1. Ulva. The $^{15}\varepsilon$ values calculated from the $\delta^{15}N$ of the algal growth and the NO_3^- show a decrease towards zero with decreasing concentration of NO_3^- (Table 3, Figure 3). At the higher concentrations, the estimate of $^{15}\varepsilon$ obtained from the algal growth (~ 3‰) and that obtained from residual NO_3^- $\delta^{15}N$ are statistically the same, while at the lower initial NO_3^- concentrations, values of $^{15}\varepsilon$ obtained from the algal $\delta^{15}N$ are significantly lower (Figure 4). If the observation that fractionation varies as a function of the concentration of NO_3^- is correct, then equation 3 can only yield a mean estimate of $^{15}\varepsilon$ as during the experiment the concentration of NO_3^- changes considerably as it is assimilated. In fact the data from the NO_3^- free drift experiment (Table 1) is best fitted by a quadratic equation confirming a change

in fractionation with changing concentration (Figure 5). Using a Chi-squared test, the improvement in the fit between the linear and non-linear model can be shown to be statistically significant at the 99% level in both the 60 and 103 μM experiments. The first differential of the quadratic equation therefore provides an estimate of ε at any value of f . Using the data from the experiments which were initiated at concentrations of 14, 60 and 103 μM NO_3^- and calculating the mean $^{15}\varepsilon$ value derived from each experiment with respect to concentration rather than f, a robust estimate of ε with respect to changing NO_3^- can be obtained (Figure 6). Data from the 500 μM experiment were not used in this estimate as a result of the small change in concentration of NO_3^- (and as a consequence a small change in f which occurred during the experiment at high concentration). Although the estimates of $^{15}\varepsilon$ obtained from the non-linear equation predict a value of less than zero at concentrations lower than ~ 1 μM, none of the experiments attained these low concentrations and therefore this observation will need to be confirmed. In addition the one syringe experiment performed with *Ulva* at a constant concentration of ~ 3 μM yielded a $^{15}\varepsilon$ value of 1 ‰, higher than the values estimated from the NO_3^- drawdown experiments. Hence such data was inconsistent with a $^{15}\varepsilon$ value below zero. In the free drift experiments however, the $\delta^{15}N$ of the measured algae was greater than that in the initial NO_3^- (5.1 and 4.0‰ in the 14 μM and 60 μM treatments respectively compared to the initial NO_3^- and algal values of 3.3 and 3.1‰ respectively) (Table 1) giving estimates of $^{15}\varepsilon$ less than zero (ε= -3.2‰). While the data of the solids appear inconsistant with the data measured on the $\delta^{15}N$ of the NO_3^-, based on previous discussion our feeling is that assimilation factors calculated from the analysis of the $\delta^{15}N$ of the NO_3^- are correct and that the $\delta^{15}N$ of the solid material might therefore be some kind of artifact as discussed earlier. Regardless of

whether $^{15}\varepsilon$ less than zero both approaches show a decrease in $^{15}\varepsilon$ with decreasing concentrations and a rate of change of appears to be greatest at the lowest concentration, i.e. between 1 and 10 μM (Figure 6). With higher concentrations (between 10- 50 to 500 μM) the fractionation appears to reach a constant positive value (ε= 3 to 4 ‰).

4.1.1.2. Agardhiella. Based on both the algal and NO_3^- $\delta^{15}N$ data, this species also exhibited a strong dependence between fractionation and NO_3^- concentration. Values of $^{15}\varepsilon$ were close to zero, or slightly negative, at low concentrations (< 10 μM) and increased between 100-500 μM reaching a value of ~ 8‰ at 500 μM (Figure 7). As a result of the fact that at most only three samples were taken for measurement of the $\delta^{15}N$ (and $\delta^{18}O$) of the NO_3^- during the free drift experiments, it was not considered valuable to fit anything more than a straight line to the data and therefore a more refined equation relating the change in ε to the concentration of NO_3^- was not calculated. As in the case of *Ulva*, there was a suggestion that $^{15}\varepsilon$ values might fall below zero at low concentrations, although the $\delta^{15}N$ of the solid material did not increase at low NO_3^- concentrations as seen in *Ulva* sp.

4.2 Concentration Dependence of the fractionation factor

In microalgae and bacteria the uptake and fractionation of NO_3^- has been proposed to be a three-step process (Granger et al., 2004; Hoch et al., 1992; Karsh et al., 2012; Karsh et al., 2014; Mariotti et al., 1982; Shearer et al., 1991). First, a transport step across the cellular membrane (ε_{in}), a nitrate reductase step (ε_{NR}) and a flux out of the cell (ε_{out}). The overall fractionation manifested by the organism, expressed as ε_{org}, is related to the influx, efflux and

nitrate reductase fractionation by equation 8 in which γ is the relative proportion of efflux relative to influx (Karsh et al., 2014).

$$\varepsilon_{org} = \varepsilon_{in} + \gamma\,(\varepsilon_{NR} + \varepsilon_{out}) \qquad (8)$$

The estimated fractionation associated with these processes in a marine diatom (*Thalassiosira weissflogii)* are; $^{15}\varepsilon_{in}$ = 2 ‰, $^{15}\varepsilon_{out}$ =1.2‰ and $^{15}\varepsilon_{NR}$ =26.6 ‰ (Karsh et al., 2012; Karsh et al., 2014). As the majority of the fractionation is associated with the NR step, the degree to which this is expressed in the external medium and also in the organism is controlled by the amount of efflux relative to influx (γ). Accepting the possibility that there may be differences between microalgae and the organisms used in this study, we have nevertheless used this model as a basis with which to explain the observations of a concentration dependence on $^{15}\varepsilon_{org}$ made in this paper. In this regard it is helpful to examine the work of Needoba et al (2004) who measured the $\delta^{15}N$ of the internal and external NO_3^- pools. They determined that the maximum difference in $\delta^{15}N$ occurred in situations in which ε_{org} was at a minimum, thus indicating that the efflux from the cell was small. Conversely when fractionation was high, the difference between the $\delta^{15}N$ of the external and internal pools was at a minimum and efflux maximal. As in both cases, the greatest potential for isotope fractionation is at the NR step (Karsh et al., 2012; Ledgard et al., 1985), the principal explanation for dependence on external concentration must relate to the ratio of NO_3^- uptake to efflux from the cell. At lower external concentrations, NO_3^- is limiting and the $\delta^{15}N$ of the internal pool is highly elevated. However, most of the NO_3^- is consumed and efflux is minimal and, although the same amount of fractionation at the NR step takes place, this isotopic signal is not communicated to the

external environment. At high concentrations the reverse is true, NO_3^- is not limiting and the fractionation experienced at the NR step is translated to the external environment. Based on our findings we propose that macroalgae may behave similarly in many respects to microalgae. However, the only study we are aware of dealing with macroalgae concluded that the concentration of NO_3^- did not influence the frationation of ^{15}N (Cohen and Fong, 2005) and would therefore appear to be in conflict with the results of this study. However, in the Cohen and Fong (2005) research the only experiments in which the concentration of NH_4^+ was not altered, in addition to NO_3^-, were carried out at relatively high concentrations of NO_3^- (> 50 uM). This is above the level at which the fractionation appeared to be constant in our study.

4.3 Oxygen Isotopic Composition of NO_3^-

The measurement of the $\delta^{18}O$ of nitrate is a relatively new technique which has helped explain both the source of NO_3^- and the mechanism of fractionation of N and O isotopes during assimilation (Granger et al., 2004; Granger et al., 2010; Leichter et al., 2007; Wankel et al., 2006; Wankel et al., 2009). The data presented here suggests that in a manner similar to ^{15}N, the fractionation of ^{18}O is dependent upon the concentration of NO_3^- in the external environment (Tables 1 and 2; Figure 8). While generally the fractionation of $\delta^{18}O$ and $\delta^{15}N$ are related in a 1:1 ratio (Granger et al., 2004), in this study, the slope of the data seem to have a value of greater than the ideal 1:1 relationship (Figure 2). It was argued by Granger et al. (2004) that this 1:1 relationship was consistent with fractionation of N and O during NR, whereas fractionation during diffusion would give a 2:1 relationship. In more recent work it was shown that there are different degrees of fractionation for N compared to O during

uptake and efflux, which would cause the relationship between $^{18}\varepsilon$ and $^{15}\varepsilon$ to rise significantly above unity, when fractionation is low (Karsh et al., 2014). Such data are in agreement with our study in that the $^{18}\varepsilon$:$^{15}\varepsilon$ ratio is closest to unity in the highest concentration (~500 μM) experiments and increases with lower initial concentrations of NO_3^- reaching a value of ~2 at 10 μM. This 2:1 relationship corresponds the lowest amount of fractionation observed ($\varepsilon \sim 0‰$). Using the rationale suggested by Granger et al (2004), this pattern is consistent with a change in fractionation from a process predominantly controlled by NR, to one in which fractionation is controlled by the relative difference between the fractionation of O and N during uptake (1.4) and efflux (2.3) (Karsh et al., 2014). If the results of these experiments are correct then the relationship between $\delta^{18}O$ and $\delta^{15}N$ also should not be linear, but rather a quadratic, similar to that observed between the $\delta^{15}N$ and concentration discussed earlier. However, as a result of the larger error on the $\delta^{18}O$ compared to $\delta^{15}N$ (0.7 vs. 0.2 ‰) this pattern was not evident in the data collected in these experiments.

4.4 Biogeochemical Implications

The observation of a concentration dependence upon ^{15}N fractionation during denitrification has been previously made for microbes (Kritee et al., 2012). Both the results of that study and the data presented here suggest that there is a relationship between fractionation and concentration during assimilation that has implications for the application of nitrogen isotopes for detection of N sources. It is clear that under typical N limiting conditions, both micro- and macroalgae have the same isotopic composition as the ambient nitrate. However, when NO_3^- concentrations are elevated, algae fractionate the external NO_3^- pool, forming biomass which is relatively isotopically more negative than the ambient NO_3^-. The residual

NO_3^- effluxed from the cell consequently becomes isotopically more positive regardless of the $\delta^{15}N$ of the original NO_3^-. Consider a hypothetical coastal estuary in which there is significant input of NO_3^- from artificial fertilizers (with a $\delta^{15}N \sim 0‰$) applied to adjacent agricultural areas. As a result of the high NO_3^- concentrations, the fractionation during assimilation by algae would be greater than zero initially producing algal material with $\delta^{15}N$ values more negative than the original NO_3^-. As the NO_3^- is consumed, the $\delta^{15}N$ of the residual NO_3^- would become more positive. Eventually isotopically positive algal material would be formed from waters which originally had a $\delta^{15}N$ close to 0‰. It might be incorrectly assumed in such instances that the algal material was affected by nitrogen derived from an isotopically positive source, when in fact the positive values were produced as a result of fractionation during assimilation.

5 Conclusions

1- There is a concentration dependence upon the fractionation of ^{15}N and ^{18}O exerted during macroalgal assimilation of NO_3^-. This dependence varies according to species, but approaches zero at low concentrations in both of the algal species studied here. This concentration dependence essentially means that in most open marine environments, which have NO_3^- concentrations of less than 2 μM, there is minimal fractionation during assimilation. In environments with higher concentrations of NO_3^-, the fractionation is greater than zero leading to enrichment in the ^{15}N of the residual NO_3^- regardless of the $\delta^{15}N$ of the original source of that NO_3^-. The observation of the concentration dependence of $^{15}\varepsilon$ helps to explain the wide range of values reported in the literature where experiments were carried over a wide range of NO_3^- concentrations.

2- The change in the $^{15}\varepsilon$ shows the largest rate of variation at low NO_3^- concentrations and there is a suggestion $^{15}\varepsilon$ may fall below zero. This might imply that organic material formed under very low NO_3^- concentrations could manifest an inverse isotopic effect.

3- The $^{18}\varepsilon$ also shows a dependence on concentration and is related to $^{15}\varepsilon$ in a 1:1 manner at higher concentrations (> 100 μM) of NO_3^-. At lower concentrations the slope of $^{18}\varepsilon$:$^{15}\varepsilon$ approaches values of 2:1.

4- The change of the fractionation of both 15N and 18O with respect to the concentration of NO_3^- supports a model in which there is a change in the origin of the fractionation from one in which the control is exerted by the NR step to one in which the control is exerted by difference between fractionation exerted in uptake and efflux.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version, at XXXXXXXXX.

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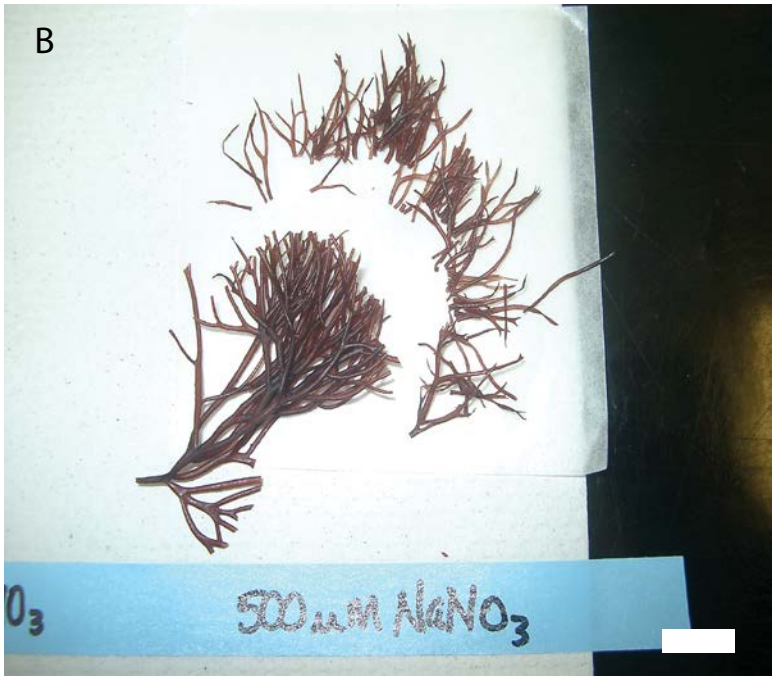


Figure 1a: Pictures showing samples of *Agardhiella* sp. grown in different concentrations of NO_3^-. From left to right, pictures show the initial individual, and specimens grown in solutions containing nominally ambient, 10 μM, 50 μM, 100μM, and 500 μM NO_3^-. All experiments in which NO_3^- was added showed approximately similar growth rates, but reduced uptake of N at lower N concentrations.

Figure 1b: At the end of the experiment the ends of the algae were trimmed and analyzed for their $\delta^{15}N$, $\delta^{13}C$, and C:N ratio. The new growth could be distinguished by comparison with the size of the original fragment (See Figure 1a) and the change in colour.

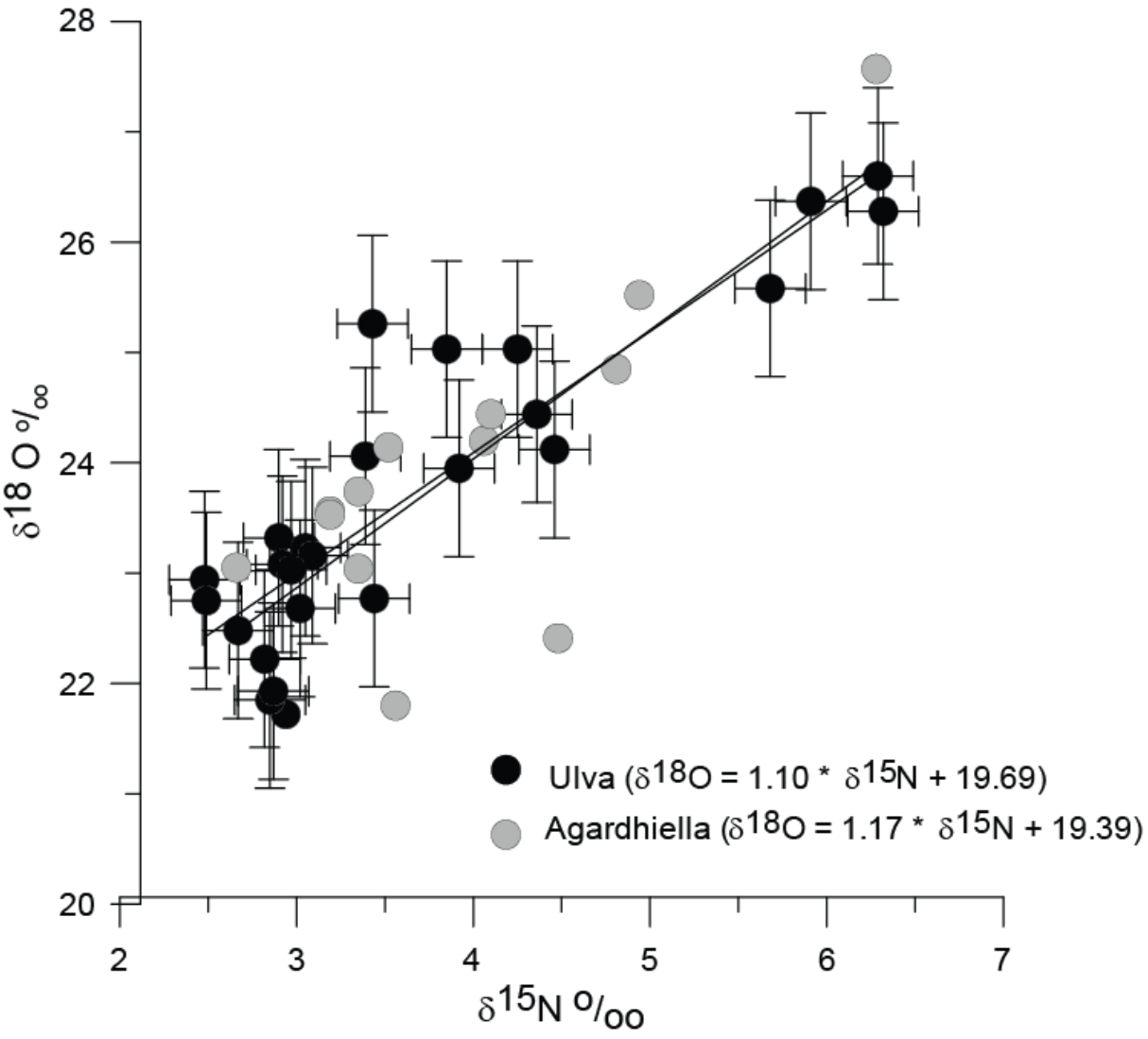


Figure 2: The relationship between $\delta^{18}O$ and $\delta^{15}N$ during the free drift experiments for the two species of algae studied. Error bars represent mean analytical error for the various analyses and have been removed for clarity from the *Agardhiella* sp. data..

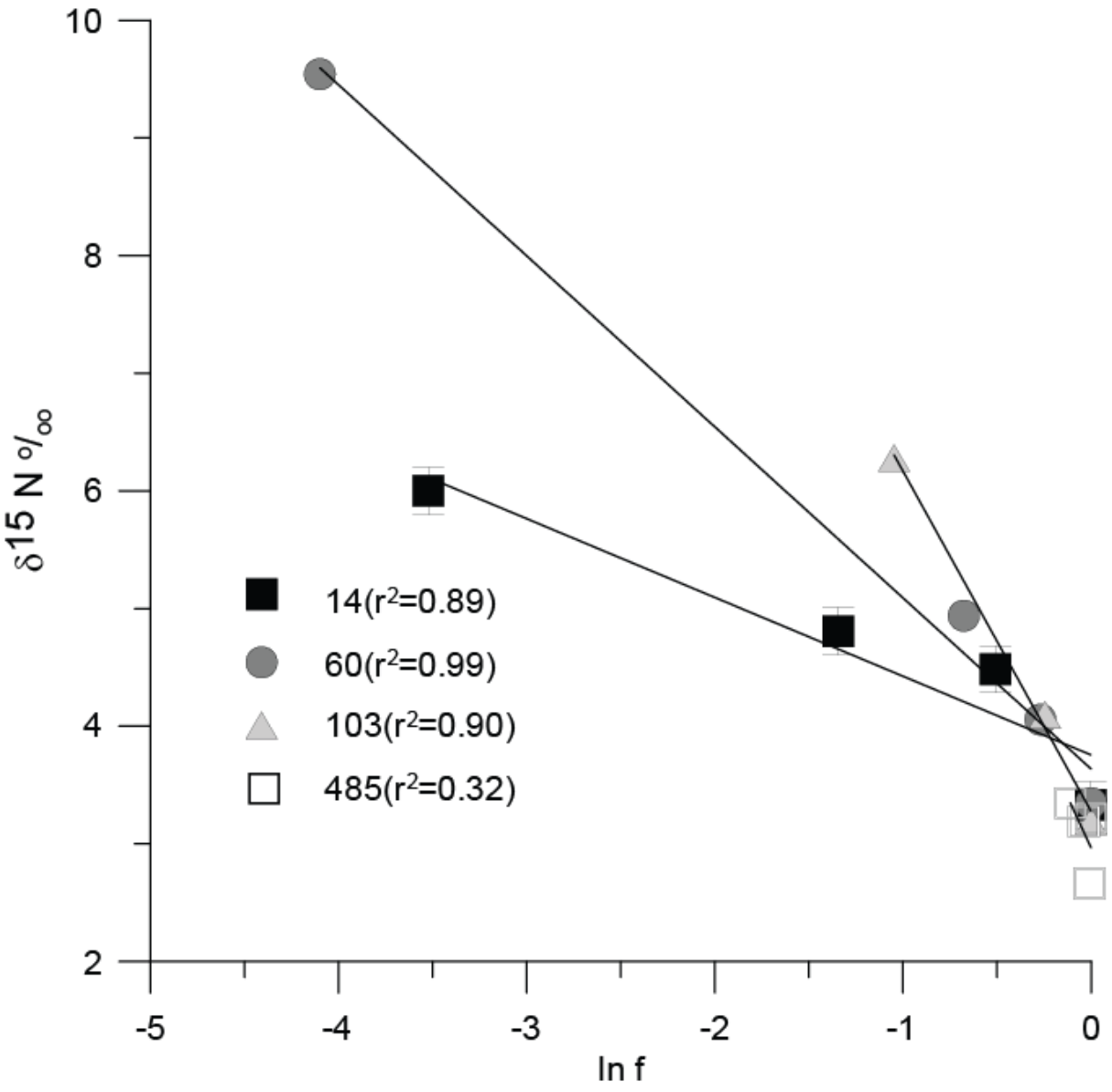


Figure 3: Calculation of $^{15}\varepsilon$ using linear regression through $\delta^{15}N$ values of NO_3^- with respect to ln f for experiments in which *Ulva* sp. was incubated for 48 hours. Samples of water were taken at 12, 24, and 48 hours and measured for the concentration of remaining NO_3^- and its $\delta^{15}N$ (and $\delta^{18}O$). The numbers refer to the different initial concentrations of NO_3^- used in each experiment (See Table 1).

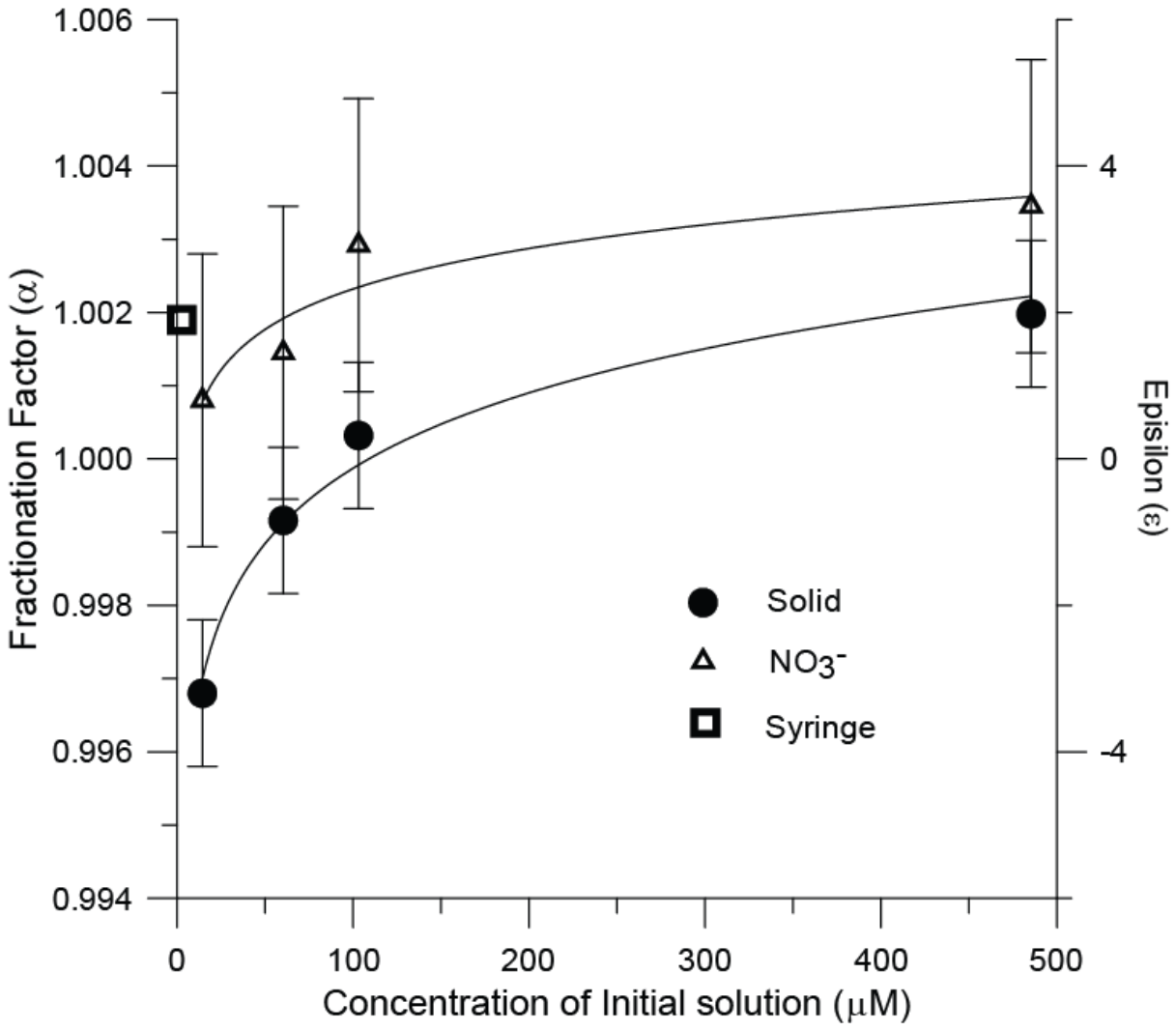


Figure 4: Estimate of fractionation factor (α) and ε during the assimilation of NO_3^- by *Ulva* sp. based on the $\delta^{15}N$ analysis of the algal material (solid) and the DIN (Data from Figure 3).

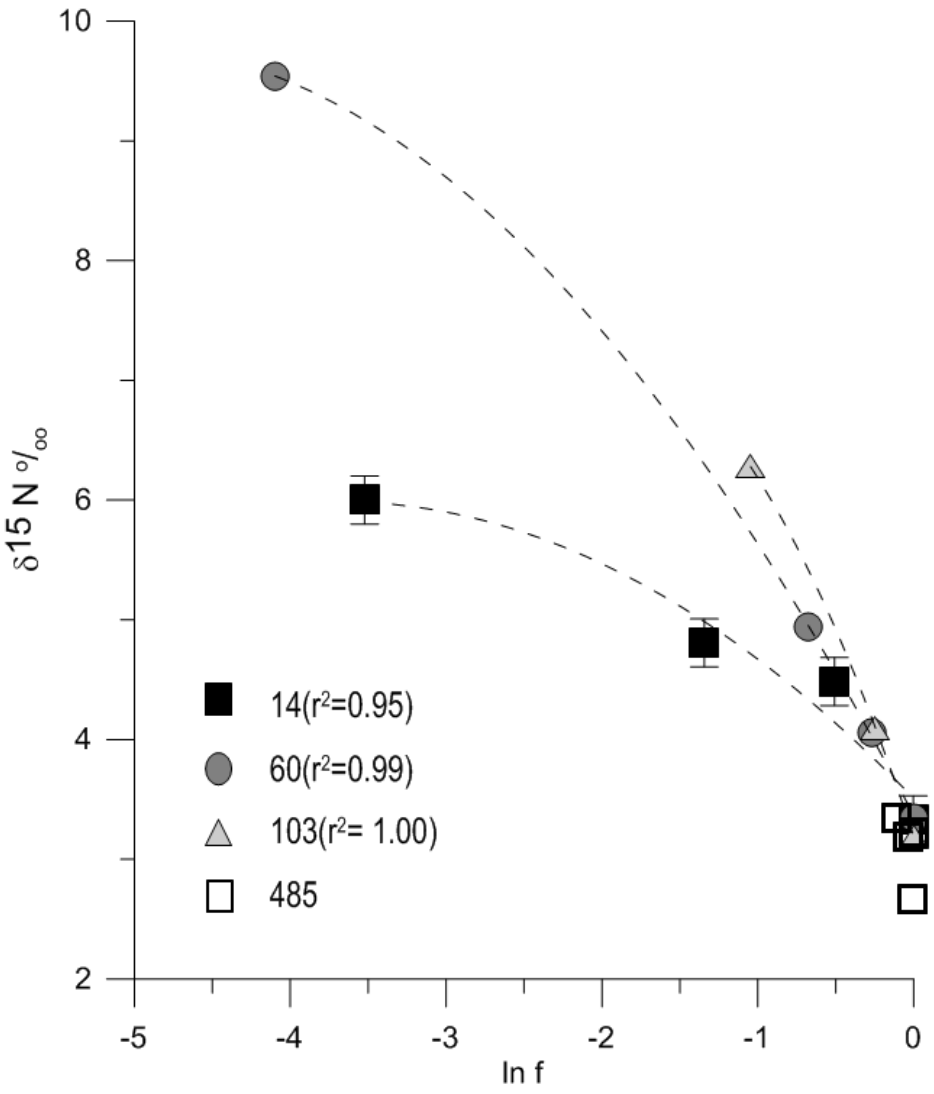
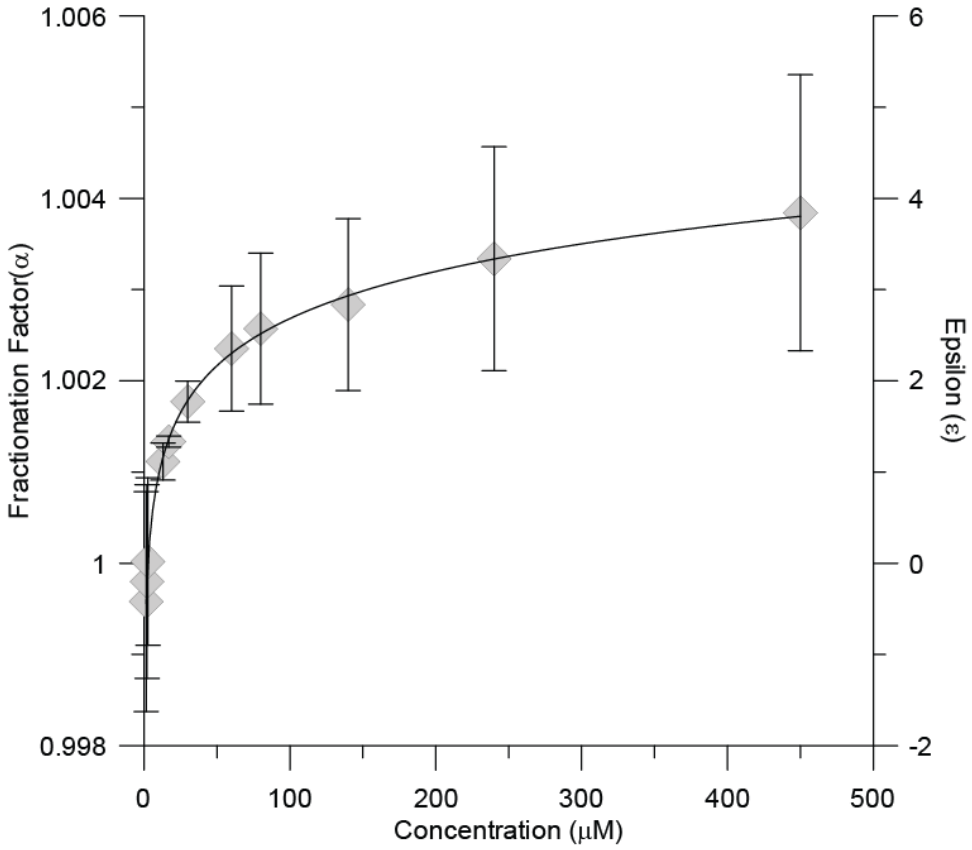


Figure 5: The changing fractionation as a consequence of decreasing NO_3^- concentration, as shown in Figure 3, necesitates the use of a non-linear curve fitting to the data. The use of a quadratic equation shows an improved fit to the data and allows the slope of the relationship to be calculated at specific concentrations using the first differential of the equation. Data from the 485 μM experiment has been omitted as a result of the small change in the f value.



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3 Figure 6: Average fractionation factors (α) and ε values calculated using the mean values
4 estimated from the first differential of the quadratic fits shown in Figure 5 for *Ulva* sp. Error
5 bars represent ± σ of the estimate calculated using equations shown Figure 5.

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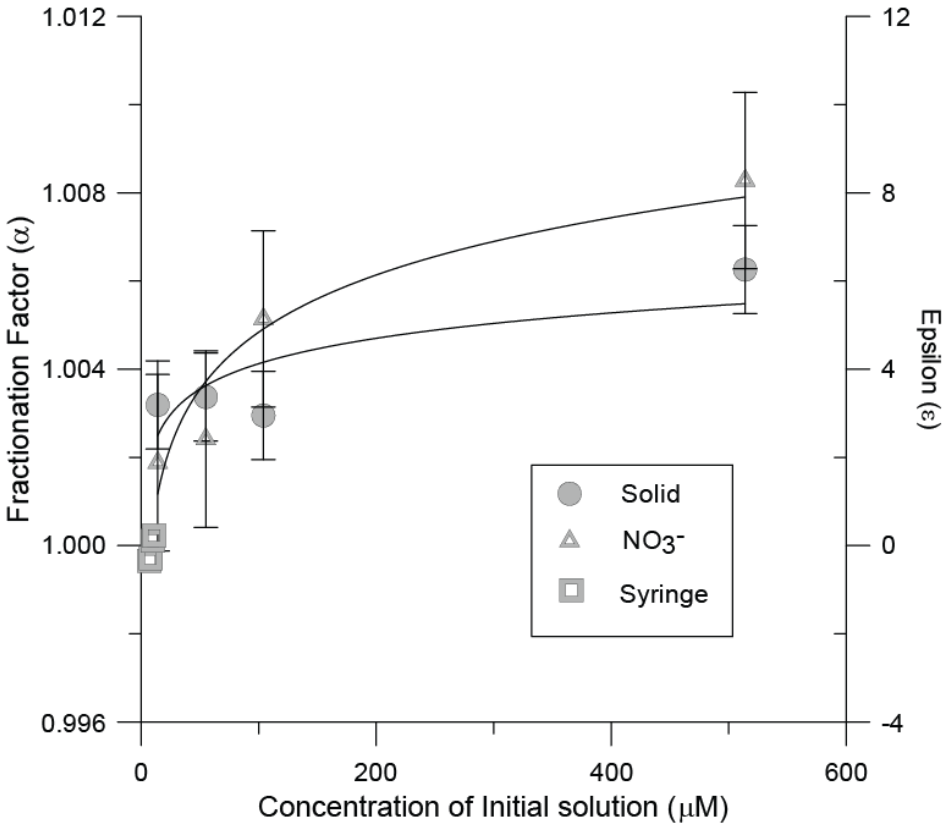

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Figure 7: Estimate of fractionation (ε) exerted during the incorporation of NO_3^- into *Agardhiella* sp. based on the $\delta^{15}N$ analysis of the algal material and the DIN. Error bars represent ± σ . of replicate measurements. The solid and the DIN data are based on the free drift results.

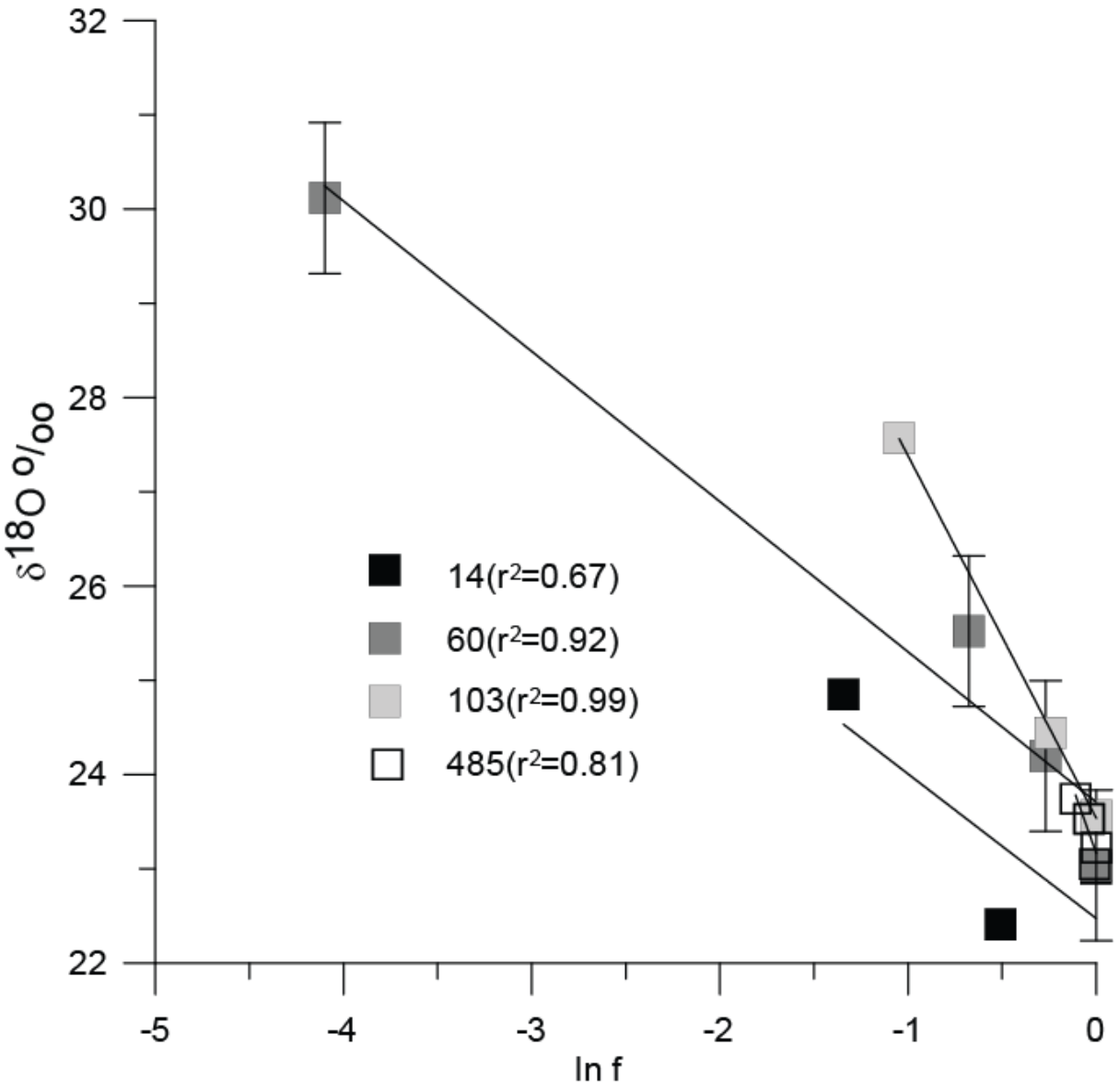

Figure 8: Relationship between the change in concentration of NO_3^- and the $\delta^{18}O$ of the NO_3^- in the *Ulva* sp. free drift experiments. Errors bars represent ± σ of the analytical precision on the $\delta^{18}O$ measurements.

Table 1: Changes in the $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- during the *Ulva* experiments.

	Ulva sp.				*Agardiella* sp.			
Time	NO_3^-	Final $\delta^{15}N$ ‰	Final $\delta^{18}O$ ‰	f	NO_3^-	Final $\delta^{15}N$ ‰	Final $\delta^{18}O$ ‰	f
Tissue	14	5.1	-	0.26	14	1.8	-	0.21
0	14	3.3	21.8	1.00	14	3.5	17.3	1.00
12	9	4.5	22.4	0.60	3	6.3	23.1	0.22
24	4	4.8	24.9	0.26	3	6.4	22.6	0.21
48	< 1	Lost	Lost	0.03	-	-	-	-
Tissue	60	6.0	-	0.51	55	1.2	-	.24
0	60	3.4	23.0	1.00	55	2.9	21.9	1.00
12	46	4.1	24.2	0.76	-	-	-	-
24	31	4.9	25.5	0.51	13	6.3	25.6	0.24
48	1	9.5	30.1	0.02	-	-	-	
Tissue	103	2.9	-	0.78	104	0.7	-	0.68
0	103	3.2	23.6	1.00	104	3.1	23.3	1.00
12	90	3.5	24.1	0.87	-	-	-	-
24	90	4.1	24.4	0.78	71	5.1	25.1	0.68
48	36	6.3	27.6	0.35	-	-	-	-
Tissue	485	1.2	-	0.99	514	-3.0	-	0.96
0	485	3.2	23.2	1.00	514	2.7	23.1	1.00
12	467	3.2	23.5	0.96	-	-	-	-
24	481	2.7	23.1	0.99	495	3.1	23.3	0.96
48	435	3.4	23.7	0.90	439	4.1	25.0	0.85

For the tissue (shaded cells) each analysis represents the mean of two replicates; for the NO_3^- each analysis represents the mean of four analyses.

Table 2: N isotopic composition of Syringe experiment algae

	NO_3 (μm)	$\delta^{15}N$ ‰	σ	*n*
Initial		3.3	0.3	16
Ulva	3	1.3	0.3	7
Agardhiella	7	3.4	0.0	2
Agardhiella	10	2.9	0.0	2

Table 3: Calculated fractionation (ε) for experiments using linear model

#	Species	NO_3^-	NO_3^-	Nitrogen	Oxygen
		μM	ε (solid) ‰	ε (DIN) ‰	ε (DIN) ‰
1	*Ulva*	2.6	2.1		
2	*Ulva*	14	-3.2	0.8	1.5
2	*Ulva*	60	-0.2	1.5	3.6
2	*Ulva*	103	0.3	2.9	3.8
2	*Ulva*	485	2.0	3.5	5.6
1	*Agardhiella*	7	0	nm	nm
1	*Agardhiella*	10	0.4	nm	nm
2	*Agardhiella*	14	3.2	1.9	nm
2	*Agardhiella*	55	3.4	2.4	2.6
2	*Agardhiella*	104	3.0	5.1	4.8
2	*Agardhiella*	514	6.3	8.3	12.9

1= Syringe experiment, 2= Free Drift; nm=not measured.